

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, G01N 33/543, 33/553 G01N 33/546	A1	(11) International Publication Number: WO 90/02205 (43) International Publication Date: 8 March 1990 (08.03.90)
---	----	--

(21) International Application Number: PCT/US89/03624 (22) International Filing Date: 23 August 1989 (23.08.89) (30) Priority data: 236,535 25 August 1988 (25.08.88) US (71) Applicant: ANGENICS, INC. [US/US]; 100 Inman Street, Cambridge, MA 02139 (US). (72) Inventor: OLSON, Jeffrey, C.; 326 Prospect Street, Unit 4, Cambridge, MA 02139 (US). (74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).	Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
---	--

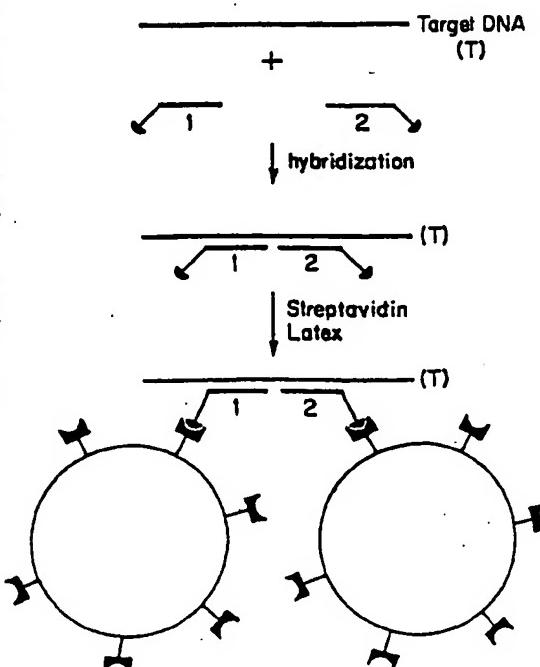
(54) Title: DETECTION OF NUCLEIC ACID SEQUENCES USING PARTICLE AGGLUTINATION

(57) Abstract

A method of detecting, identifying and/or quantitating nucleic acids in a sample through determination of agglutination or inhibition of agglutination of suspendable particles having a member of a specific binding pair bound thereto is described. The member of the specific binding pair can be bound directly to the particle surfaces or attached through a spacer molecule which can, in turn, be either covalently bound or adsorbed to the particle surfaces. The suspendable particles are small enough to remain in suspension and will generally have a large particle size relative to the molecular weight of the DNA or RNA which is being determined. The presence or absence of nucleic acid sequences in a sample is determined by detecting agglutination of particles having a member of a specific binding pair bound thereto which becomes crosslinked via nucleic acid sequence complex labelled with at least two molecules of one member of a specific binding pair spaced an appropriate distance apart that the complex can act as a bridge between at least two particles labelled with a second member of a specific binding pair.

Key:

→ Biotin
 Streptavidin



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	L	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LJ	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

-1-

DETECTION OF NUCLEIC ACID SEQUENCES USING
PARTICLE AGGLUTINATION

Description

Technical Field

05 This invention is in the field of ligand assays and in particular relates to the detection and quantification of nucleic acid sequences through nucleic acid hybridization.

Background Information

10 Nucleic acid hybridization is the basis for many methods used for the detection and identification of nucleic acids in a sample. Hybridization is the process by which a single stranded nucleic acid (i.e., DNA or RNA) recognizes its complementary 15 strand and hydrogen bonds to it, forming a double stranded molecule. That is, when single stranded nucleic acids are combined under appropriate conditions, complementary base sequences pair and double-stranded hybrid molecules are formed.

20 In nucleic acid hybridization assays (e.g., DNA-DNA, DNA-RNA), it is often the case that sample DNA or RNA is attached to a solid support (e.g., a cellulose nitrate filter) by simply allowing it to adhere to the support. A labelled probe DNA or RNA 25 is then added under conditions appropriate for hybridization of complementary sequences to occur.

-2-

The presence of sequences complementary to the probe sequence is determined by detecting binding of the labelled probe to bound (sample) DNA or RNA.

Attachment of DNA to a solid support can be
05 accomplished by non-specific physical adsorption of single stranded nucleic acid (e.g., to nitrocellulose papers) and by chemical bonding (e.g., to agarose/Sepharose, aminoethyl-Sepharose, Sephadexes, cellulose).

10 Nucleic acid hybridization provides a very sensitive and specific approach to detecting and identifying nucleic acids in samples. However, methods presently available require enzyme - or radioactive tracer - labelled nucleic acid probes,
15 time-consuming procedures and/or sophisticated equipment. Presently, nucleic acid hybrids are detected by observing a change in the absorbance of a DNA solution; by physically isolating hybridized DNA from nonhybridized DNA using chromatography or hydroxyapatite and quantitating the hybridized DNA;
20 or by capturing the hybridized DNA on nitrocellulose. Generally, these methods require labelled nucleic acids because, although a nucleic acid sequence will anneal only with its complementary sequence, the presence of hybrid double stranded molecules is undetectable unless the probe is labelled. For example, nucleic acid sequences are often radioactively labelled using phosphorous 25 (³²P), which can be introduced into DNA molecules

-3-

as phosphate groups while they are being synthesized by host bacteria or by an in vitro reaction. Radioactively labelled nucleic acid sequences are widely used, but radioactive material can pose a risk to
05 the user. Such materials typically have short half-lives and, therefore, limited shelf lives. In addition, expensive, sophisticated equipment is necessary for their detection.

In European Patent Office (EPO) Application
10 0,130,523, Dattagupta and Crothers describe a solid support for nucleic acids and an immobilized nucleic acid probe capable of hybridizing with complementary nucleic acids. The solid support, to which a
nucleic acid can be bound by irradiation, is described as comprising a solid substrate which has
15 reactive groups; a photochemically reactive intercalator compound or other ligand capable of binding nucleic acids; and a divalent radical chemically linking the solid and the nucleic acid binding ligand. Upon photoactivation, the ligands chemically link with nucleic acids. Specifically, the solid substrate is nitrocellulose paper having hydroxyl groups and linked by a bifunctional reagent to an amino-substituted compound, which in turn is
20 photochemically linked to a nucleic acid. The resulting immobilized nucleic acid is described as being useful in hybridization assays in which the support with coupled DNA is combined with a sample
25

-4-

to be assayed for the occurrence of sequences complementary to those on the support and a detection (labelled) probe. Testing the solid support for presence of a label (e.g., radioactivity) shows 05 whether hybridization has occurred or not (and thus whether complementary DNA is present).

In EPO Application 0,130,515, Dattagupta et al. describe a method for detecting the presence in a sample of a particular nucleic acid sequence which 10 involves dual nucleic acid hybridization. A sample containing unknown DNA is mixed with two nucleic acid probes which are complementary to two nonoverlapping portions of the nucleic acid sequence to be detected. One probe is labelled and soluble in the 15 sample and the other probe is fixed to a solid support (e.g., nitrocellulose). The mixture is allowed to stand under hybridizing conditions; hybridization to both probes by DNA in the sample occurs only if it contains sequences complementary 20 to both probes. Separating the dual hybridization product (by separating the solid support) and detecting the label attached to it is said to provide a method of determining the presence in a sample of the DNA sequence of interest.

25 In U.S. Patent 4,486,539, Ranki and Soderlund describe a kit for use in detecting and identifying viral or bacterial nucleic acids. The one-step sandwich hybridization procedure on which the kit is based requires two nucleic acid fragments which have

-5-

no sequences in common, and are complementary to two regions of the genome of the microbe to be identified. One fragment is fixed to a solid carrier (e.g., a nitrocellulose filter) and the
05 other is labelled. Contact between nucleic acids to be identified and nucleic acids on the solid carrier results in annealing of complementary base pairs to form a hybrid. Annealing the second (labelled) fragment to the fragment to be identified results in
10 labelling of those fragments formed on the solid support and thus allows their detection and quantification.

In Patent Cooperation Treaty (PCT) WO84/02721, Kohne describes a method for detecting and quantifying bacteria and viruses containing RNA. After the
15 nucleic acids in a sample and a probe (radioactively labelled nucleic acid sequences complementary to the RNA of the organism to be detected) have been incubated under hybridization conditions, the degree
20 of hybridization with the marked probe is measured. The method is described as being useful for hybridization in solution or hybridization with an immobilized nucleic acid probe.

In Biochemistry, Vol. 16, No. 7, (1977),
25 Manning et al. teach a method of gene enrichment based on the avidin-biotin interaction. Biotin is covalently coupled to cytochrome c using an N-hydroxysuccinimide ester acylation. This modified cytochrome c is covalently attached to the RNA via

-6-

formaldehyde crosslinks. The RNA-cytochrome c-biotin is then hybridized to long single strands of DNA. Manning et al. teach several alternative separation steps for separating the DNA:RNA:biotin hybrids from unhybridized DNA. First, avidin is covalently attached to water soluble poly(methyl methacrylate) spheres by a carbodiimide condensation. The biotin sites on the hybrids bind to the spheres and the mixture is then banded in cesium chloride. The spheres function as floats for the DNA strands to which they are attached and, therefore, band at a low density relative to the unattached DNA strands. A second alternative step would be the use of conventional affinity chromatography wherein avidin is attached to a solid support and the mixture containing DNA:RNA:biotin and unhybridized DNA is slowly passed through a column of this material. Third, removal of the DNA from either the above DNA:RNA:avidin:sphere complex or the DNA:RNA:avidin:bead column is accomplished by denaturation of the hybrid with sodium hydroxide.

In copending application Serial No. 836,100, filed March 4, 1986, Gefter et al. describe a method of detecting nucleic acid sequences using particle agglutination. A direct assaying method is described wherein if a sample contains nucleic acid sequences complementary to those attached to the solid support, hybridization will occur and cause particle agglutination. Alternatively, Gefter et

-7-

05 a1. describe an indirect assaying method wherein inhibition of agglutination can be used to detect the presence of nucleic acid sequences of interest in a sample. In this case, two different nucleic acid sequences (e.g., + and -) are attached to the solid support; that is, some of the solid support particles have (+) strands attached to them and others, (-) strands. If the sample contains nucleic acids complementary to either of the attached 10 sequences, agglutination of the solid support is inhibited. In either case, detection of the degree of agglutination can be carried out visually or by another method known in the art. The degree of agglutination is indicative of the extent of hybridization of complementary nucleic acid sequences, which is, in turn, indicative of the presence of 15 nucleic acid sequences in the sample.

20 At the present time, there is a need for a method of detecting the presence of nucleic acid sequences in biological samples which has the specificity of nucleic acid hybridization techniques, but does not require the use of radioactive materials, time-consuming preparation and sophisticated equipment.

25 Disclosure of the Invention

The present invention is a method of determining the presence or absence of nucleic acid sequences of interest (DNA or RNA) in a sample. The

-8-

method is particularly useful because it does not require the use of radioactive reagents or sophisticated equipment, and it can be carried out rapidly. In one embodiment, a sample to be assayed for nucleic acid sequences of interest (target nucleic acid sequences) is combined with: 1) two mutually noncomplementary probes, which can hybridize to substantially non-overlapping regions of the target nucleic acid sequence, each probe comprising a nucleic acid sequence labelled with at least one molecule of a first member of a specific binding pair (SBP₁) and 2) particles having affixed to their surfaces the second member of the specific binding pair (SBP₂). These components are combined under conditions appropriate for hybridization of complementary nucleic acid sequences and binding of the members of the specific binding pair to occur. If target nucleic acid sequences are present in the sample, hybridization of complementary sequences occurs, resulting in formation of a two (probe - first member of a specific binding pair) - target nucleic acid sequence (hereinafter 2(probe SBP₁)-TNA complex. In the 2(probe - SBP₁)-TNA complex, the molecules of the first member of a specific binding pair must be spaced an appropriate distance apart so that the complex can act as a bridge between at least two particles labelled with the second member of a specific binding pair. An appropriate distance is defined as a distance between two molecules of SBP₁ which makes it

-9-

possible for the complex to act as a bridge between two particles labelled with SBP₂. This distance will vary depending on reactions conditions, and choice of a specific binding pair and can be determined
05 empirically for a particular set of conditions.

Binding of the SBP₁ present in the 2(probe - SBP₁)-TNA complexes with the second member of the SBP₂ present on particles results in agglutination of the particles. If target nucleic acid sequences
10 are not present in the sample, hybridization does not occur, the particles do not become crosslinked and there is no agglutination. In the method of the present invention in which the quantity of target nucleic acid sequence is to be determined, the
15 number or size of agglutinated particles or degree of agglutination is determined and compared with a predetermined relationship (or standard) between the number or size of agglutinated particles and the quantity of target nucleic acid sequence.

20 In a second embodiment, a sample to be assayed for target nucleic acid sequences is combined with:
1) two mutually noncomplementary primers, one of which is a nucleic acid sequence complementary to a region of one strand of the target nucleic acid
25 sequence and the other of which is a nucleic acid sequence complementary to a different region of the opposite strand of the target nucleic acid sequence (i.e., a region other than that to which the first primer is complementary) and each of which is

-10-

labelled with at least one molecule of one member of a specific binding pair; 2) an appropriately selected polymerase; 3) appropriately selected nucleotides; and 4) particles having affixed to their surfaces the second member of the specific binding pair, under conditions appropriate for hybridization, amplification and binding of the two SBP members to occur. If target nucleic acid sequences are present in the sample, labelled primer 1 hybridizes to the target nucleic acid sequence and initiates the polymerization of a nucleic acid sequence, resulting in extension or elongation of the primer nucleic acid sequence. The double stranded nucleic acid sequences formed from this process are then denatured. Labelled primer 1 hybridizes to a first region of the target nucleic acid sequence in the same manner as in the previous step; labelled primer 2 hybridizes to a second region of the newly-synthesized nucleic acid sequence. Labelled primers 1 and 2 then initiate polymerization of the appropriate nucleic acid sequences. This process results in the amplification of a target nucleic acid sequence and the formation of a 2(primer - one member of a specific binding pair target)-target nucleic acid sequence complex (hereinafter 2(primer - SBP₁)-TNA). It is repeated until a sufficient quantity of the target nucleic acid sequence is present (in the sample). Enzymes which can be used in the method of the present invention are E. coli

-11-

DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, Taq polymerase, or any polymerase capable of polymerizing nucleic acid sequences.

05 The amplification method used in this second embodiment is specifically modified to result in labelling of the primers with one member of a specific binding pair. Thus, the target nucleic acid sequence is simultaneously amplified and
10 labelled in such a way that a 2(primer - SBP₁)-TNA complex is formed. The molecules of the first member of the specific binding pair present in the complex must be spaced an appropriate distance apart so that the complex can act as a bridge between at
15 least two particles having the second member of a specific binding pair affixed to their surfaces. If target nucleic acid sequences are present in the sample, hybridization of complementary sequences and amplification occurs, resulting in the formation of
20 a 2(primer - SBP₁)-TNA complex. Binding of the SBP₁ present on the 2(primer - SBP₁)-TNA complexes with the second member of the SBP (i.e., SBP₂) present on the particles results in the agglutination of the particles. If target nucleic acid sequences are not
25 present in the sample, hybridization and amplification do not occur, the particles do not become crosslinked and there is no agglutination. In the method of the present invention in which the quantity of target nucleic acid sequence is to be

-12-

determined, the number or value of agglutinated particles is determined and compared with a predetermined relationship (or standard) between the number or size of agglutinated particles and the 05 quantity of target nucleic acid sequence. An example of an amplification method which can be modified to incorporate an appropriate label for use in this second embodiment is the polymerase chain reaction described in U.S. Patent 4,683,195, 10 issued July 28, 1987. Any amplification method which can be modified to incorporate an appropriate label into the nucleic acid sequence being amplified can be used in the present invention.

15 In both embodiments of this invention, the hybridization and agglutination steps can occur sequentially or simultaneously. That is, appropriate reactants can be combined sequentially or can be added simultaneously to a single reaction vessel. Examples of specific binding pairs might 20 include biotin and avidin or streptavidin, antibodies and their corresponding antigens, and receptors and their corresponding ligands.

The method of this invention has very broad application, both in terms of the types of samples 25 for which it is useful and the types of organisms which can be detected in such samples. The presence of a particular nucleic acid sequence in any type of biological sample (e.g., blood and other tissues; urine; and foodstuffs such as milk) can be

-13-

determined using the present invention, for instance. The presence of bacteria and viruses in biological samples can be detected using particle agglutination, for example. In addition, because 05 bacteria have common nucleic acid sequences, as well as sequences specific to a strain or class within the species, it is possible to detect all bacteria in a sample by using a shared nucleic acid sequence or to detect specific bacteria by using a nucleic 10 acid sequence unique to that strain or class.

Brief Description of the Drawings

Figure 1 is a schematic representation of one embodiment of constructing the 2(probe - SBP₁)-TNA complex, in which two singly biotinylated nucleic 15 acid probes complementary to two different regions of the target nucleic acid are allowed to hybridize with the target nucleic acid sequence.

Figure 2(a) is a schematic representation of the second embodiment of constructing the 2(primer - SBP₁)-TNA complex, wherein the polymerase chain 20 reaction amplification method is modified to incorporate labelling of the primers with one member of a specific binding pair.

Figure 2(b) is a schematic representation of the 2(primer - SBP₁)-TNA complex formed in a modified amplification method acting as a bridge 25 between microparticles labelled with a second member of a specific binding pair.

-14-

Detailed Description of the Invention

The present invention is based on the discovery
that 2(probe - SBP₁)-TNA or 2(primer - SBP₁)-TNA
complexes which are labelled with at least two
05 molecules of one member of a specific binding pair
(e.g. biotin) can, if the labelling molecules are
separated by an appropriate distance, be used to
crosslink particles, preferably microparticles
(e.g., latex), labelled with the corresponding
10 second member of the specific binding pair (e.g.,
avidin).

The present invention also is based on the fact
that agglutination or crosslinking of particles can
be used as a readout signal for the detection of a
15 target nucleic acid sequence, since 2(probe -
SBP₁)-TNA or 2(primer - SBP₁)-TNA, capable of
forming bridges between at least two particles, will
not be formed in the absence of the target nucleic
acid sequence. For ease of discussion, biotin and
20 avidin will be used in the discussion below.
However, it is in no way intended to limit the
invention to this particular specific binding pair:

In one embodiment, the present invention
comprises several steps which may be carried out in
25 sequence or simultaneously. First, the sample to be
assayed for nucleic acid sequences of interest is
combined with two mutually noncomplementary probes
which can hybridize to substantially non-overlapping
regions of the target nucleic acid sequence, each
30 probe comprising a nucleic acid sequence labelled

-15-

with at least one biotin molecule and subjected to
conditions appropriate for hybridization of
complementary nucleic acid sequences to occur. The
probes are selected so that they hybridize to two
05 substantially non-overlapping regions of the target
nucleic acid in such a way that a 2(probe-Biotin)-
TNA complex is created. The 2(probe-Biotin)-TNA
complex contains at least two biotin molecules
spaced an appropriate distance apart so that the
10 complex can act as a bridge between at least two
avidin labelled particles. An appropriate distance
is defined as a distance far enough apart that the
complex can act as a bridge between two particles
labelled with SBP₂. This distance will vary
15 depending on reaction conditions and choice of a
specific binding pair and can be determined
empirically for a particular set of conditions.

In the second step, the 2(probe-Biotin)-TNA
complexes are combined with avidin-labelled
20 particles under conditions appropriate for the
binding of biotin and avidin. Binding of the biotin
present in the probe-Biotin-TNA complex with the
avidin present on the particles results in
agglutination of the particles. If target nucleic
acid sequences are not present in the sample,
25 hybridization does not occur, the particles do not
become crosslinked and there is no agglutination.

In a second embodiment, the sample which is to
be assayed for target nucleic acid sequences is
30 combined with two biotin labelled mutually

-16-

noncomplementary primers, a polymerase and selected nucleotides, under conditions appropriate for hybridization of complementary nucleic acid sequences and nucleic acid sequence amplification.

05 If target nucleic acid sequences are present in the sample, labelled primer 1 hybridizes to the target nucleic acid sequences and initiates the polymerization of a complementary nucleic acid strand by elongating the primer nucleic acid

10 sequence. The newly synthesized double stranded nucleic acid molecule is denatured and biotinylated primer 1 hybridizes in the same manner as in the previous step and biotinylated primer 2 hybridizes to a second region of the newly synthesized

15 complementary nucleic acid strand. Biotinylated primers 1 and 2 then initiate polymerization of their appropriate nucleic acid sequences. This process, which results in both the amplification of a target nucleic acid sequence and the formation of

20 a 2(primer-Biotin)-TNA complex, is repeated until a sufficient quantity of target nucleic acid is present. Enzymes which are useful within the present invention are E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase, T4 DNA

25 polymerase, Taq polymerase, or any polymerase capable of polymerizing nucleic acid sequences. The modified amplification method forms a 2(primer-Biotin)-TNA complex which includes at least two molecules of biotin spaced an appropriate

-17-

distance apart so that the complex can act as a bridge between two avidin labelled particles. The primers of this embodiment serve a function analogous to that of the probes of the first 05 embodiment (i.e., they selectively hybridize to the target nucleic acid sequence). However, in addition to serving as a probe, the primer, in combination with an enzyme, is involved in the synthesis of a complementary nucleic acid strand. In a second 10 step, the 2(primer-Biotin)-TNA complexes are combined with avidin-labelled particles, under conditions appropriate for the binding of biotin and avidin. Binding of the biotin present in the 15 2(primer-Biotin)-TNA complex with the avidin present on the particles results in agglutination of the particles. If target nucleic acid sequences are not present in the sample, hybridization does not occur, amplification does not occur, the particles do not crosslink and there is no agglutination. The 20 modified amplification method which is useful within the present invention is any method which specifically amplifies a target nucleic acid sequence while simultaneously labelling the sequence with at least two molecules of one member of a 25 specific binding pair.

-18-

The 2(Probe-Specific Binding Pair₁)-Target Nucleic Acid Sequence or 2(Primer-Specific Binding Pair₁)-Target Nucleic Acid Sequence Complexes

A 2(probe - SBP₁)-TNA or 2(primer - SBP₁)-TNA comprises hybridized nucleic acid sequences which contain at least two molecules of one member of a specific binding pair spaced an appropriate distance apart so that the sequence can act as a bridge between at least two particles coated with the second member of the specific binding pair and causing crosslinking or agglutination. One example of a 2(probe - SBP₁)-TNA or 2(primer - SBP₁)-TNA complex is a double stranded DNA sequence which is labelled with one biotin molecule at each end, in such a manner that avidin-coated particles can be bridged by this complex sequence. More than one biotin can be used to label each end of a complex, provided that the biotin molecules at one end of the sequence are not so numerous that they can act as crosslinkers themselves with avidin-labelled particles. The distance between the sites of labelling must be sufficiently great that bridging can occur. The distance appropriate for reaction conditions and specific binding pairs used vary and can be determined empirically for each reaction process. For example, double stranded DNA sequences containing biotin spaced from 30-250 bp apart can and have been used to cause agglutination of avidin latex under various conditions. In addition, less

-19-

than 30 or more than 250 bp might be appropriate for use under some conditions; the effectiveness of such distances can be determined empirically.

Another example of a 2(probe - SBP₁)-TNA or 2(primer - SBP₁)-TNA is a sequence with biotin molecules interspersed along the length of the sequence. For example, such a sequence could be created in the course of amplification by the incorporation of biotinylated nucleotides into an elongating nucleic acid strand. Such a complex, if sufficiently long, is able to bridge at least two avidin-coated particles.

Production of 2(Probe-SBP₁)-TNA or
2(Primer-SBP₁)-TNA Complexes

2(probe-SBP₁)-TNA or 2(primer - SBP₁)-TNA complexes must be produced in such a way that their existence in a reaction is a product of the presence of target nucleic acid sequences in the sample. In addition, the starting materials required for the production of 2(probe-SBP₁)-TNA or 2(primer-SBP₁)-TNA complexes must not, themselves, be capable of causing crosslinking of particles. Avidin and biotin will again be used below for ease of discussion. However, the present invention is in no way intended to be limited to this specific binding pair, since any specific binding pair can be used in the present invention.

In one method of producing a 2(probe-Biotin)-TNA complex, two mutually noncomplementary nucleic

-20-

acid sequences each of which bears at least one biotin molecule complementary to two different appropriately spaced regions of the target nucleic acid sequence (DNA or RNA) are combined with a
05 sample to be assayed, under conditions appropriate for hybridization with target nucleic acid sequences. This results in a 2(probe - SBP₁)-TNA, a hybridized polynucleotide molecule labelled with at least two spacially separated biotins, which can act
10 as a bridge between particles because of the interaction of biotin with avidin present on avidin-coated particles. Unhybridized probes cause no crosslinking or agglutination because they contain either a single biotin or a series of biotins which
15 are too close together to form a bridge between microparticles. Thus, agglutination of the particles can be used to detect the presence of target nucleic acid sequences in the sample.

In one method of producing a 2(primer-Biotin)-TNA complex a modified amplification method is used
20 to produce additional copies of the target nucleic acid sequences and to label the amplified target nucleic acid sequences with biotin. An amplification method which can be modified to be
25 useful in the present invention is any method which amplifies small quantities of the desired nucleic acid segment and incorporates biotin labelling. For example, the polymerase chain reaction procedure (PCR), described in U.S. Patent 4,683,195, issued
30 July 28, 1987, hereby incorporated by reference, can

-21-

be used within the present invention if modified to incorporate biotin labelling. When used in the method of the present invention, the PCR procedure results in the production of a 2(primer-Biotin)TNA complex which is capable of crosslinking particles labelled with avidin. (See Figure 2). The PCR procedure amplifies specific regions of a nucleic acid sequence by the use of two short mutually non-complementary oligonucleotide primers that will hybridize to opposite strands on adjacent sides of a region of the target nucleic acid sequence in such a way to delineate a sequence to be amplified. The first step of the PCR method is heating of the sample to denature the target nucleic acid. The sample is then cooled in the presence of an excess of two different oligonucleotide primers. The first primer (hereinafter Primer 1) hybridizes to a region of the target nucleic acid (hereinafter DNA T). In the presence of nucleotides and DNA polymerase a new nucleic acid strand complementary to DNA T will be synthesized using Primer 1 (hereinafter DNA T'). This newly synthesized double nucleic acid strand (DNA T - DNA T') is then denatured and a new primer 1 is allowed to hybridize to the DNA T and a second primer (herein Primer 2) is allowed to hybridize to DNA T'. DNA polymerase once again synthesizes new DNA T and DNA T' strands from Primers 1 and 2. The sample is heated once again, causing denaturation of the DNA. Four new primers (i.e., two Primer 1's and two Primer 2's) are allowed to hybridize to the two

-22-

DNA T strands and the two DNA T' strands,
respectively, allowing the next round of synthesis
by DNA polymerase to occur. This process is repeated
until there is a sufficient quantity of target
05 nucleic acid sequence present to be detected. Each
double stranded fragment produced will contain mers
1 and 2 incorporated into them. If primers 1 and 2
were originally biotinylated, a 2(primer-
Biotin)-TNA complex is created in the reaction.
10 This 2(primer - SBP₁)-TNA complex can then be used
to cause agglutination of avidin-coated latex
particles.

The present invention has many advantages over
the presently-available methods. Most importantly,
15 the assay is nonisotopic and nonenzymatic which
increases both the safety and convenience of
handling and the shelflife of reagents. Another
advantage is that the hybridization step occurs in
solution, giving assays performed by this method a
20 kinetic advantage over a solid support test.
Hybridization in solution occurs more rapidly than
hybridization reactions wherein one reactant is
attached to a solid support. Further, single-
stranded biotinylated probes do not participate in
25 the agglutination reaction. Thus, an excess amount
of probe can be added which also increases the
kinetics of the hybridization.

This assay is very specific for a particular
nucleic acid sequence because it is necessary for
30 two probes to hybridize to the target nucleic acid

-23-

sequence instead of one probe. In addition, these two probes have to hybridize within a certain distance (approximately 250 base pairs or less) of each other. Single probe sequences hybridizing 05 inappropriately will not be detected. Another advantage is that there is no requirement for separation of the unhybridized and hybridized probes, as unhybridized probes do not participate in the agglutination reaction.

10 The avidin affixed to particles can be covalently bound directly to the particle or covalently or non-covalently bound to a spacer molecule which can, in turn, be either covalently bound or adsorbed to the particle surfaces.

15 Nucleic Acid Segments

Deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) sequences used as the probe can be in a solution which is contacted with the sample nucleic acid strands. The nucleic acid sequences chosen as 20 probes are mutually non-complementary to each other, appropriately specific to the target nucleic acid sequence, and hybridize to the target nucleic acid sequence in such a way that the molecules of the (SBP₁) are spaced an appropriate distance apart. Any gene or nucleic acid sequence (DNA or RNA) of 25 interest can be used as the target nucleic acid sequence. For example, target nucleic acid sequences can be sequences complementary to a ribosomal RNA sequence that is present in either all

-24-

bacteria (inclusive) or sequences complementary to a ribosomal RNA sequence that is characteristic of a single type of bacteria (exclusive). If inclusive ribosomal nucleic acid sequences are used as the probes, contact with a sample containing RNA from any bacteria will result in hybridization of complementary sequences, crosslinking, and agglutination of the avidin-bearing particles. If exclusive ribosomal probes are used, agglutination will occur only if RNA from the specific type of bacteria is present. Because RNA is rapidly degraded when an organism dies, only viable cells will be detected. In the first case, all viable bacteria in a sample will be detected; in the second, only the one type of viable bacteria having RNA complementary to the probes selected will be detected. This could be used, for example, in detecting viable bacteria in a milk sample pre-treated to release bacterial RNA; it provides a simple, rapid and specific alternative to the standard plate count presently used in the dairy industry.

The nucleic acid sequences to be used as probes can be of almost any length, provided that they are labelled in such a way that a hybridization reaction will generate crosslinking sequences. It has been demonstrated that a stable bond or hybrid is formed when complementary nucleic acid sequences have five or more bases. Generally, therefore, the nucleic acid sequences used as probes will be five or more bases in length.

-25-

Nucleic acid sequences in solution for use as probes can be obtained by cloning of isolated DNA or RNA segments according to methods well known in the art. See, for example, Maniatis, T. et al.,

05 Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982). For example, any appropriate restriction enzyme can be used to remove/excise a DNA fragment to be used as a probe from a naturally occurring source. Bacterial DNA
10 can be cleaved at selected sites on either side of the DNA fragment to be used as a probe; the resulting probe fragments can be isolated from other fragments (and thus purified) electrophoretically. The isolated DNA fragments can then be amplified by
15 inserting them into a plasmid or a bacterial virus (bacteriophage), which is in turn inserted into an appropriate bacterial host cell. As the cells containing the plasmid proliferate, the plasmid also replicates, producing many copies of the DNA
20 fragment to be used as a probe. After the cells have been allowed to proliferate, the hybrid plasmids are isolated and purified, resulting in the isolation of many copies of the DNA fragment to be used as a probe.

25 Nucleic acid sequences for use as probes can also be generated synthetically or, if they occur in nature in sufficient quantities, simply by isolation and purification.

-26-

Identifying, Quantitating or Isolating Target
Nucleic Acid Sequences

The method of the present invention can be used to identify, quantitate or isolate a target nucleic acid sequence. The present method can be used as a screening technique for determining the presence or absence of a target nucleic acid sequence in a sample by observing whether agglutination of particles occurs (i.e., agglutination (+); no agglutination (-)). The quantity of a target nucleic acid sequence in a sample can be determined by comparing the number or size of the agglutinated particles with a predetermined relationship (or standard) between the number or size of agglutinated particles and the quantity of target nucleic acid. A target nucleic acid sequence can also be isolated from a sample using the present method by separating particles labelled with one member of a specific binding pair having the target nucleic acid sequences bound thereto from the rest of the sample. The nucleic acid sequences are removed from the particles, if desired, using known techniques. An example of a device which would be useful for identifying, quantitating or isolating the target nucleic acid sequence in the present invention would be the capillary flow device described by Cox et al. in co-pending application Serial No. 189,983, filed May 4, 1988, hereby incorporated by reference.

-27-

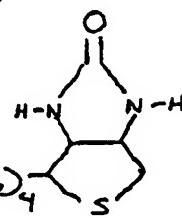
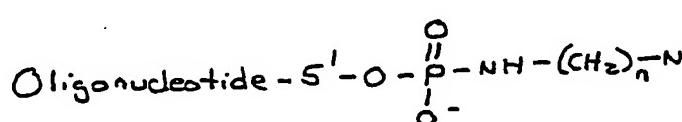
Labelling Nucleic Acid Sequences with One Member of
a SBP

Biotin is used as an example of a first member
of a SBP in the discussion below. However, it is in
05 no way intended to limit the invention to this
particular specific binding pair.

The biotinylated nucleic acid sequences used as
probes or primers within the present invention can
be purchased from Synthetic Genetics or prepared
10 using any method which attaches biotin to the
nucleic acid sequence in a manner which allows the
probe or primer to be part of a crosslinking
sequence. An example of a method which is useful
15 for the biotin labelling of the probes or primers of
the present invention is described by Chu et al.,
(DNA, Vol. 4, No. 4, (1985), pp. 327-331). In the
method of Chu et al., a deoxyribonucleotide sequence
is synthesized using the solid-phase phosphoramidite
method. The deoxyribonucleotide oligomer was
20 converted to a 5'-phosphorylated oligomer by
allowing 2.5 A₂₅₄ units of oligonucleotide to react
with ATP and 15 units of T₄ polynucleotide kinase
for 75 minutes at 37°C in 80 ul of kinase buffer
(Maniatis et al., Molecular Cloning, (1982)) at pH
25 7.6 containing 0.2 mM ATP. Phosphorylated oligo-
nucleotides were separated from non-phosphorylated
oligonucleotides by HPLC at pH 12 on a RPC-5 column.
The 5'- phospho-oligonucleotide is converted to the
30 5'- phosphoroimidazolide by treatment with 0.12 M 1-
ethyl-3,3,dimethylaminopropyl carbodiimide in 0.1 ml

-28-

of 0.1M imidazole HCl buffer at pH 6.1 for one hour at room temperature. 5'-phosphoroimidazolide oligonucleotide is separated from the 5'-phosphorylated oligonucleotide by HPLC on a RPC-5 column. The 5'-phosphoroimidazolide oligonucleotide is then converted to the 5'-ethylenediamine oligonucleotide by treatment with 0.25 M ethylenediamine at pH 7.7 for one hour at 50°C. The ethylenediamine oligonucleotide is purified by HPLC on a RPC-5 column and dialyzed against 0.2 M Hepes buffer (pH 7.7). N-Hydroxysuccinimidobiotin (5 mg/ml) is then added to solutions of the ethylenediamine oligonucleotides (0.001-0.1A₂₆₀ units/ml in 0.2 M Hepes buffer at pH 7.7) and allowed to react at room temperature for one hour. The biotinylated oligonucleotide was obtained via HPLC on RPC-5 column or by electrophoresis on a non-denaturing 20% polyacrylamide gel at pH 8. The biotin labelled nucleic acid sequence has the following chemical formula:



Solid Support Materials

For ease of discussion avidin is used as an example of the second member of the specific binding pair in the discussion below. However, it is in no way intended to limit the invention to this specific

-29-

binding pair. In the discussion below, avidin is intended to encompass both avidin and streptavidin.

The solid support to which the avidin is attached can be essentially any finely divided
05 insoluble material to which avidin can be covalently attached or irreversibly adsorbed; that is, the material must be reactive (in a covalent or noncovalent manner) with avidin or must be adsorbed to or covalently bound to a substance, referred to
10 as a spacer molecule, which can be covalently or noncovalently linked to the avidin. The solid support can be, for example, latex, charcoal, colloidal gold, bentonite or glass. In addition, silica gel, controlled pore glass, red blood cells
- 15 and liposomes can be used. In fact, any such particle to which avidin can be attached can be employed in the method of the present invention, provided that after attachment of avidin, it can be made to remain in a colloidal suspension in the
20 absence of the nucleic acid sequence of interest, and that its agglutination in the presence of the nucleic acid sequence of interest can be detected either visually or instrumentally. Glass or other particles can be derivatized to form reactive
25 functional groups (see, for example, Weetall, U.S. Patent 3,652,761; Koster, et al., Tetrahedron Letters 24: 747 (1983)) capable of reacting with avidin.

The solid support need not have a particular shape (configuration), but will often be spherical.
30

-30-

It must be small enough to remain in suspension and will generally have a large particle size relative to the molecular weight of the DNA or RNA probe (e.g., less than 100 microns).

05 Conditions Appropriate for Effecting the
Agglutination of Particles

Kinetic energy is necessary for agglutination of the particles to occur. The kinetic energy causes particles coated with a member of a specific binding pair to collide with each other or come within a close proximity of the 2(probe - SBP₁) -TNA complexes or 2(primer - SBP₁) -TNA complexes labelled with a second member of a specific binding pair which allows the two members of the specific binding pair to bind. This kinetic energy can be provided from an external source (i.e., by shaking, rocking, or stirring) or it may be inherently provided within the reaction process (i.e., via capillary flow, for example).

20 Measurement of Agglutination or Aggregation of
Avidin Particles Having Bound Biotinylated DNA or
RNA as an Indicator of the Presence of Target
Nucleic Acid Sequences in a Sample

Determination of agglutination or aggregation of avidin particles having bound biotinylated DNA or RNA can be carried out by any method capable of detecting the degree of agglutination present after

-31-

sample and probe(s) have been contacted under conditions appropriate for hybridization to occur, and the labelled target nucleic acid sequences have been contacted with avidin-coated microparticles under 05 conditions appropriate for agglutination to occur. For example, detection can be carried out visually using the unaided eye (e.g., visualization against a black or other dark background); microscopically; or by turbidimetric measurements. In addition, a 10 particle counter having a size threshhold can be used to detect aggregated/unaggregated particles. Selective counting techniques, which are well known in the art, make it possible to count the number of particles in a given size range and thus allow 15 quantitative assays to be carried out. See, for example, U.S. Patent 4,184,849 to C.L. Cambiaso et al., in which such techniques are described. It is also possible to use a filter having a defined pore 20 size; the pore size is selected so as to allow nonaggregated particles to pass through but to prevent aggregated particles from doing so. See, for example, U.S. Patent 4,459,361 to M.L. Gefter.

When low numbers of particles must be detected, it is possible, using known techniques, to amplify 25 the detectability of the particles through selection of such properties as color, refractive index, optical density and fluorescence. The particles can be enzyme labelled in such a way that the enzymes

-32-

attached to the particle surfaces catalyze a color-producing change (thus aiding particle detection). Such amplification techniques are particularly useful when the nucleic acid sequences 05 of interest are present in a sample in low concentrations.

Determination of agglutination can also be carried out using the device described by Cottingham in U.S. Patent 4,597,944 issued July 1, 1986.

10 Cottingham teaches an agglutination reagent detection system for controlling and detecting an agglutination reaction without dilution of the agglutination reagent. The detection system of Cottingham comprises a sample field cell for 15 receiving agglutination reagents and defining an isoplanar-sample field, a light source directed towards the sample field, and a detector which detects a change in the amount of light from the sample field caused by the agglutination reaction. 20 The device of Cottingham provides a reproducible and sensitive detection of the agglutination which occurs in the sample field.

Determination of agglutination can also be carried out using the device described by Cox et al. 25 in co-pending application Serial No. 189,983, filed May 4, 1988, the teachings of which are hereby incorporated by reference. Cox et al. describe a capillary flow device which uses capillary flow to

-33-

cause particles to collide with each other,
ultimately leading to agglutination of particles. In
the present invention, avidin would be attached to
the particles, which would then be capable of
05 crosslinking with a 2(probe - SBP₁) -TNA complex
incorporating the first member of the specific
binding pair, i.e., biotin.

The device of Cox et al. uses two types of
particles, one magnetic and the other non-magnetic
10 and detectable. Magnetic particles are removed from
the reacted mixture, at a defined location on the
capillary track, by magnetic attraction, with the
result that any particles remaining in the reacted
mixture are non-magnetic detectable nonagglutinated
15 particles. The occurrence and/or number of
non-magnetic particles is subsequently determined,
using known methods, and serves as an indication of
presence/ absence of the analyte of interest and/or
as the basis for determining the quantity of analyte
20 present. In the method of the present invention in
which the quantity of the target nucleic acid
sequence is determined, the number or volume of
non-magnetic particles recovered is determined and
compared with a predetermined relationship (or
25 standard) between the number or volume of
non-magnetic particles and the quantity of nucleic
acid sequence of interest.

An agglutinographic slide can also be used in
the method of the present invention as a means for
30 producing and detecting or measuring agglutination.

-34-

See U.S. Patent 4,597,944, issued July 1, 1986, for an example. An agglutinographic slide can include two panels which are spaced far enough apart to effect a capillary action between them. A channel, 05 located between the two panels, transports liquids from the entrance end of the slide to the viewing end of the slide. The capillary flow in the channel causes particles to collide, thus, allowing agglutination to occur. The channel can be longer 10 than the slide which increases the reaction time, thus, agglutinations occur which are larger and therefore easier to detect visually.

This invention will now be more specifically described by the following examples, which are not 15 intended to be limiting in any way.

Attachment of Streptavidin or Avidin to Latex

A 10% (w/v) Seragen 0.716 micron carboxy modified latex is diluted in distilled water to 4% (w/v). The latex is then spun at 25,000x g for 15 20 minutes at 25°C. The supernatent is removed and the pellet is resuspended in distilled water to 4% (w/v). The two previous steps are repeated twice except that after the third spin the pellet is resuspended in distilled water to 10% (w/v).

25 A 1-hydroxybenzotriazole hydrate (NHB) stock solution is made by dissolving 0.1 gram of NHB in 1.6 ml dimethylformamide. 2.4 ml of distilled water is added to this solution which results in a stock

-35-

solution with a concentration of 1.85×10^{-4} moles/ml of NHB. A 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide methyl-P-toluene sulfonate (CMC) stock solution is made by dissolving 05 0.105 grams of CMC in 2 mls of distilled water. This stock solution has a concentration of 1.18×10^{-4} moles/ml of CMC.

A three molar excess of NHB stock solution (3 moles NHB/1 mole carboxy group) is added dropwise to 10 the rapidly stirring 10% (w/v) carboxylated latex. The latex/NHB solution is left to stir at 4°C for 10 minutes. A 3 molar excess of CMC stock solution (3 moles NHB/1 mole carboxyl group) is added dropwise to the latex/NHB solution and left to stir at 4°C 15 for 90 minutes. After 90 minutes, the latex is diluted to 4% (w/v) in 0.1 M NaCl. The latex is spun at 25,000 x g for fifteen minutes at 4°C. The supernatent is removed and the pellet resuspended to 4% (w/v) in 0.1 M NaCl. The two previous steps are 20 repeated four times and the latex is resuspended to 4% (w/v) in 0.1 M NaCl.

An equal volume of streptavidin, or avidin, at the appropriate concentration, which is dissolved in 0.1 M KPO₄ (pH 8.5), is added to the 4% (w/v) latex 25 while stirring. The latex-streptavidin/avidin solution is left to stir overnight at room temperature. The solution is then spun at 25,000 x g at 25°C for 15 minutes. The supernatent is discarded and the pellet is resuspended in 0.5M NaCl and 0.5%

-36-

sodium dodecylsulfate (SDS) solution to 4% (w/v). This solution is kept at 37°C for one hour. After the hour, the solution is spun at 25,000 x g for 15 minutes at 25°C. The supernatant is again discarded
05 and the pellet is resuspended in distilled water to a dilution of 4% (w/v). The two previous steps are repeated four times. Finally the latex is resuspended in distilled water to 4% (w/v) and 0.01% methiolate and stored at 4°C.

10 Using the Polymerase Chain Reaction to Create a
2 (Primer-Biotin)-TNA Complex Capable of Effecting
Agglutination of Streptavidin Latex

- Reagents used in the polymerase chain reaction (PCR) procedure for amplification of DNA to cause
15 agglutination of avidin or streptavidin latex
- a. M13mp18 single stranded DNA - New England Biolabs
 - b. Biotinylated primer 1601 1/2 - Synthetic Genetics
 - c. Biotinylated primer 1601 2/2 - Synthetic Genetics
 - d. Thermus Aquaticus polymerase (Taq) - New England Biolabs
 - e. Deoxynucleotide triphosphates (dATP, dCTP, dGTP, TTP) - Cetus-Perkin Elmer
 - f. Bovine Serum Albumin (BSA) Fraction V - Miles

-37-

- g. Taq Polymerase Buffer (10x)
 - 1. 500 mM KCl
 - 2. 100 mM Tris-Cl pH 8.3
 - 3. 15 mM MgCl₂
- 05 h. Agglutination buffer (2x)
 - 1. 0.1M Tris-Cl pH8.0
 - 2. 2% Polyethylene glycol 8000 (PEG)
 - 3. 0.2m NaCl
 - 4. 0.4 (w/v) Methyl cellulose 4000
- 10 i. Non-biotinylated primer #1224 - New England
Biolabs
- j. Non-biotinylated primer #1201 - New England
Biolabs

Location and Sequence of Primers used in PCR

Procedures

M13mp18 5' TCGTATGTTGTGGAATTGTGAGCGGATAACAATTCACACAGGAAACAGCTATGACCATG
15 1201 - AACAGCTATGACCATG
1601 2/2 - ACAGCTATGACCATG
B
ATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCA
20 AGCTTGGCACTGGCCGTCGTTTACAACGTCGTGACTGGGAAAACCCCTGGCG - 3'
1224 - CAGCACTGACCCCTTTGGGACCGC
1601 1/2 - CAGCACTGACCCCTT - 5'
B

In addition to the reagents listed in Table 1,
the following reagents were added to each of the
25 test tubes: a. 10.0 ul of 10X Taq polymerase
buffer, b. 20.0 ul of BSA (1 mg/ml), and c. 2.0 ul
of each deoxynucleotide triphosphate (final
concentration of each dNTP = 200 uM).

-38-

TABLE 1

	Test Tubes	uL Added (Final conc.)	1	2	3	4	5
05	M13mpl8	uL Added (Final conc.)	4.0 2.5×10^{11} strands	4.0 $(2.5 \times 10^{11}$ strands)	--	10.0 $(10^5$ strands)	10.00 $(10^5$ strands)
10	Biotin Primer 1601 1/2	uL Added (Final conc.)	2.52 (0.4uM)	2.52 (0.4uM)	2.52 (0.4uM)	--	2.52 (0.4uM)
15	Biotin Primer 1602 2/2	uL Added (Final conc.)	3.08 (0.4uM)	3.08 (0.4uM)	3.08 (0.4uM)	--	3.08 (0.4uM)
20	Non-Biotin Primer 1224	uL Added (Final conc.)	--	--	--	0.64 (0.4uM)	--
	Non-Biotin Primer 1201	uL Added (Final conc.)	--	--	--	0.42 (0.4uM)	--
	Taq Polymerase	uL Added (Final conc.)	1.0 (2.5 units)	--	1.0 (2.5 units)	1.0 (2.5 units)	1.0 (2.5 units)

Distilled water was added to all of the test tubes to bring the total volume to 100 ul. The values in parentheses are the final concentrations of the solutions.

-39-

During the first cycle, all the tubes (i.e. 1-5) were treated by heating to 94°C for two minutes, cooling two minutes at 37°C and then three minutes at 67°C. During subsequent cycles, the 05 tubes were treated by heating to 94°C for one minute, 37°C for two minutes, and 67°C for three minutes. 1uL (2.5 units) of Taq polymerase was added to appropriate tubes after each ten cycles. During the last cycle before an aliquot of the PCR 10 reaction was to be tested, the 67°C step was extended to ten minutes. The aliquot was then placed at room temperature for ten minutes before testing.

The samples were tested by removing 25 ul of 15 the PCR reaction, mixing this 25 ul with 50 ul of 2x agglutination buffer and then 25 ul of 4% (w/v) streptavidin latex prepared as described above. The contents of the tube were mixed by pipetting up and down six times and then examining the sample using 20 the agglutinographic slide. The viewing window was read as positive agglutination (+) or nonagglutination (-). The results of the PCR reactions taken after varied number of cycles is recorded below.

-40-

TABLE 2

Test Tubes	12 Cycles	30 Cycles	40 Cycles
1) No Taq polymerase 10 ¹¹ M13mpl8 strands 05 Biotinylated primers	-	not tested	not tested
2) Taq polymerase 10 ¹¹ M13mpl8 strands 10 Biotinylated primers	+	not tested	not tested
3) Taq polymerase No M13mpl8 strands 15 Biotinylated primers	-	-	-
4) Taq polymerase 10 ⁵ M13mpl8 strands 20 Non-biotinylated primers	not tested	-	-
5) Taq polymerase 10 ⁵ M13mpl8 strands Biotinylated primers	not tested	-	+

-41-

Next, 25 uL from the PCR reaction tubes 4 and 5 were run on a 1.5% agarose gel. Both lanes showed a band at approximately 120 bp which is the size of the band that is expected to be created in the PCR reaction.

The results from these experiments confirm that the use of a modified version of the PCR procedure using biotinylated primers, i.e., instead of unlabelled primers, will produce 2(primer-Biotin)TNA complexes capable of crosslinking with avidin or streptavidin-bearing particles causing agglutination to occur. Further, it is clear from the controls, i.e., tubes which did not contain M13 mp18 target DNA or Taq polymerase or which contained nonbiotinylated primers, that the target DNA has to be present, the primers have to be biotinylated and there must be production of an amplified nucleic acid sequence for agglutination to occur.

Industrial Applicability

Nucleic acid probes and hybridization assays according to this invention have a variety of possible applications in which the ability to detect, quantify and/or identify complementary nucleic acid sequences of interest in biological samples of all kinds is of great value. For example, they are useful in a research context as tools for studying gene structure and inheritance. In addition, they are useful in clinical settings

-42-

for the detection and identification of infectious agents and for prenatal diagnosis of genetic disorders. Finally, DNA probes have utility in the diagnosis of cancer (by providing information on the structure of oncogenes); in tissue typing; in veterinary and plant diagnostics; and in food testing (by providing a quicker, more convenient means of testing for the presence of pathogens).

05

Equivalents

10

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

15

-43-

CLAIMS

1. A method of determining the presence or absence of a target nucleic acid sequences in a sample, comprising:
 - a. combining the sample with 1) at least two mutually non-complementary probes which are nucleic acid sequences which can hybridize to two substantially non-overlapping areas of the target nucleic acid sequence wherein each probe is labelled with at least one molecule of a first member of a specific binding pair and 2) particles having affixed thereto the second member of a specific binding pair, under conditions appropriate for hybridization of complementary nucleic acid sequences and binding of the two members of the specific binding pair resulting in agglutination of the particles; and
 - b. detecting agglutination.
2. A method according to Claim 1, wherein the first member of the specific binding pair is biotin and the second member of the specific binding pair is avidin or streptavidin.

-44-

3. The method according to Claim 1, further comprising identifying, isolating, or quantitating the target nucleic acid sequence.
4. A method according to Claim 1, wherein the
05 first member of the specific binding pair is an antibody and the second member is an antigen, or the first member of the specific binding pair is an antigen and the second member of the specific binding pair is an antibody.
- 10 5. A method according to Claim 1, wherein the first member of the specific binding pair is a receptor and the second member of the specific binding pair is a ligand capable of binding to the receptor or the first member of the
15 specific binding pair is a ligand capable of binding to the receptor and the second member of the specific binding pair is the receptor.
- 20 6. A method according to Claim 1, wherein the second member of the specific binding pair is covalently bound to the particles or covalently or noncovalently bound to a second material which is adsorbed to or covalently bound to the particles.

-45-

7. A method according to Claim 1, wherein the particles are selected from the group consisting of latex particles, charcoal particles, colloidal gold, bentonite particles and glass particles.
8. A method of determining the presence or absence of a target nucleic acid sequence in a sample, comprising the steps of:
 - a. combining: 1) the sample; 2) at least two mutually non-complementary probes which are nucleic acid sequences which can hybridize to two substantially non-overlapping areas of the target nucleic acid sequence and wherein each probe is labelled with at least one molecule of a first member of a specific binding pair; 3) magnetic particles having affixed thereto the second member of the specific binding pair; and 4) non-magnetic particles having affixed thereto the second member of the specific binding pair, to produce a reaction mixture, in a sample receptacle of a capillary flow device comprising:
 - 1) a capillary track having a first and a second distal end; 2) a sample receptacle in communication with the first distal end of the capillary track; and 3) a liquid reservoir communication;

-46-

- b. incubating the reaction mixture under conditions appropriate for the binding of the specific binding pair;
 - c. separating the magnetic particles from the non-magnetic particles by applying a magnetic force to the reacted mixture; and
 - d. determining the presence or absence of non-magnetic particles.
9. The method according to Claim 8, wherein the capillary flow device further comprises a particle concentrator in communication with the second distal end of the capillary track.
10. A method of determining the presence or absence of a target nucleic acid sequence in a sample, comprising:
- a. combining the sample with 1) at least two mutually non-complementary probes which are a nucleic acid sequence which can hybridize to two substantially non-overlapping areas of the target nucleic acid sequence and wherein each probe is labelled with at least one molecule of a first member of a specific binding pair, under conditions appropriate for hybridization to occur, and 2)

-47-

- 05 particles having affixed thereto the second member of the specific binding pair, to produce a reaction mixture in a sample receptacle of an agglutinographic slide device comprising: 1) a first panel and a second panel spaced apart to define a chamber therebetween; 2) a sample receptacle and 3) a viewing chamber wherein the entrance opening and the viewing chamber are connected by the chamber;
- 10 b. incubating the reaction mixture under conditions appropriate for the binding of the specific binding pair; and
- 15 c. determining the presence or absence of agglutination by examining the reaction mixture through the viewing chamber.
11. A method of determining the presence or absence of a target nucleic acid in a sample, comprising:
- 20 a. combining: 1) the sample; 2) two mutually non-complementary primers, one of which is a nucleic acid sequence complementary to a first region of one strand of the target nucleic acid and is labelled with a first member of a specific binding pair; 3) a second primer which is a nucleic acid sequence which is complementary to a
- 25

-48-

- second region of the opposite strand of target nucleic acid and is labelled with the first member of the specific binding pair; 4) at least one appropriately selected polymerase; and 5) appropriate selected nucleotides, under conditions appropriate for amplification and labelling of target nucleic acid sequences to form 2(SBP₁-primer)-target nucleic acid sequence complexes;
- 05 b. combining complexes formed in step a) with particles having affixed thereto the second member of the specific binding pair, under conditions appropriate for binding of the two members of the specific binding pair, resulting in agglutination of the particles; and
- 10 c. detecting agglutination.
- 15
12. A method according to Claim 11, wherein the first member of the specific binding pair is biotin and the second member of the specific binding pair is avidin or streptavidin.
- 20
13. The method according to Claim 11, further comprising identifying, isolating, or quantitating the target nucleic acid sequence.
- 25

-49-

14. A method according to Claim 11, wherein the first member of the specific binding pair is an antibody and the second member is an antigen, or the first member of the specific binding
05 pair is an antigen and the second member of the specific binding pair is an antibody.
15. A method according to Claim 11, wherein the first member of the specific binding pair is a receptor and the second member of the specific
10 binding pair is a ligand capable of binding to the receptor or the first member of the specific binding pair is a ligand capable of binding to the receptor and the second member of the specific binding pair is the receptor.
- 15 16. A method according to Claim 11, wherein the second member of the specific binding pair is covalently bound to the particles or covalently or noncovalently bound to a second material which is absorbed to or covalently bound to the
20 particles.
17. A method according to Claim 11, wherein the particles are selected from the group consisting of latex particles, charcoal particles, colloidal gold, bentonite particles
25 and glass particles.

-50-

18. A method of determining the presence or absence
of a target nucleic acid sequence in a sample,
comprising the steps of:
05 a. combining: 1) the sample; 2) two mutually
non-complementary primers, one of which is
a nucleic acid sequence complementary to a
first region of one strand of the target
nucleic acid and is labelled with a first
member of a specific binding pair; 3) a
10 second primer which is a nucleic acid
sequence which is complementary to a
second region of the opposite strand of
the target nucleic acid sequence and is
labelled with the first member of the
15 specific binding pair; 4) at least one
appropriately selected polymerase; 5)
appropriately selected nucleotides; 6)
magnetic particles having affixed thereto
the second member of the specific binding
pair; and 7) non-magnetic particles having
20 affixed thereto the second member of the
specific binding pair, to produce a
reaction mixture, in a sample receptacle
of a capillary flow device comprising: 1)
25 a capillary track having a first and a
second distal end; 2) a sample receptacle
in communication with the first distal end
of the capillary track; and 3) a liquid
reservoir;

-51-

- b. incubating the reaction mixture under conditions appropriate for the binding of the specific binding pair;
 - c. separating the magnetic particles from the non-magnetic particles by applying a magnetic force to the reacted mixture;
 - d. capturing the non-magnetic particles on a solid material; and
 - e. determining the presence or absence of captured non-magnetic particles.
- 05
- 10
- 15 19. A method according to Claim 18, wherein the capillary flow device further comprises a particle concentrator in communication with the second distal end of the capillary track.
- 20 20. A method of determining the presence or absence of a target nucleic acid sequence in a sample, comprising:
 - a. combining the sample with 1) two mutually non-complementary primers, one of which is a nucleic acid sequence complementary to a first region of one strand of the target nucleic acid and is labelled with a first member of a specific binding pair; 2) a second primer which is a nucleic acid

-52-

- sequence which is complementary to a second region of the opposite strand of the target nucleic acid and is labelled with the first member of the specific binding pair; 3) at least one appropriately selected polymerase; 4) appropriately selected nucleotides; and 5) particles having affixed thereto the second member of the specific binding pair, to produce a reaction mixture in a sample receptacle of an agglutinographic slide device comprising: 1) a first panel and a second panel spaced apart to define a chamber therebetween,
- 15 2) a sample receptacle and 3) a viewing chamber wherein the entrance opening and the viewing chamber are connected by the chamber;
- b. incubating the reaction mixture under conditions appropriate for the binding of the specific binding pair; and
- 20 c. determining the presence or absence of agglutination by examining the reaction mixture through the viewing chamber.

-53-

21. A kit for the detection of nucleic acid sequences in a sample by detecting agglutination, comprising:
 - a. a container;
 - 05 b. at least two mutually non-complementary probes which are nucleic acid sequences of the target nucleic acid sequence, and wherein each probe is labelled with at least one molecule of a first member of a specific binding pair; and
 - 10 c. particles having affixed thereto the second member of a specific binding pair.
22. A kit according to Claim 21, wherein the first member of the specific binding pair is biotin and the second member of the specific binding pair is avidin or streptavidin.
15
23. A kit according to Claim 21, wherein the first member of the specific binding pair is an antibody and the second member is an antigen, or the first member of the specific binding pair is an antigen and the second member of the specific binding pair is an antibody.
20
24. A kit according to Claim 21, wherein the first member of the specific binding pair is a receptor and the second member of the specific binding pair is a ligand capable of binding to the receptor or the first member of the
25

-54-

specific binding pair is a ligand capable of binding to the receptor and the second member of the specific binding pair is the receptor.

25. A kit according to Claim 21, wherein the second
05 member of the specific binding pair is covalently bound to the particles or covalently or noncovalently bound to a second material which is adsorbed to or covalently bound to the particles.
- 10 26. The kit according to Claim 21, wherein the particles are selected from the group consisting of latex particles, charcoal particles, colloidal gold, bentonite particles and glass particles.
- 15 27. A kit for the detection of nucleic acid sequences in a sample by detecting agglutination, comprising:
 - a. a container;
 - b. at least two mutually non-complementary primers, one of which is a nucleic acid sequence complementary to a first region of the opposite strand of the target nucleic acid and is labelled with a first member of a specific binding pair; and
 - 20 a second primer which is a nucleic acid sequence which is complementary to a second region of the opposite strand of

-55-

- the target nucleic acid and is labelled with the first member of the specific binding pair;
- 05 c. at least one appropriately selected polymerase;
- d. appropriate selected nucleotides; and
- e. particles having affixed thereto the second member of a specific binding pair.
- 10 28. A kit according to Claim 27, wherein the first member of the specific binding pair is biotin and the second member of the specific binding pair is avidin or streptavidin.
- 15 29. A kit according to Claim 27, wherein the first member of the specific binding pair is an antibody and the second member is an antigen, or the first member of the specific binding pair is an antigen and the second member of the specific binding pair is an antibody.
- 20 30. A kit according to Claim 27, wherein the first member of the specific binding pair is a receptor and the second member of the specific binding pair is a ligand capable of binding to the receptor or the first member of the specific binding is a ligand capable of binding to the receptor and the second member of the specific binding pair is the receptor.
- 25

-56-

31. A kit according to Claim 27, wherein the second member of the specific binding pair is covalently bound to the particles or covalently or noncovalently bound to a second material which is adsorbed to or covalently bound to the particles.
- 05

Key:

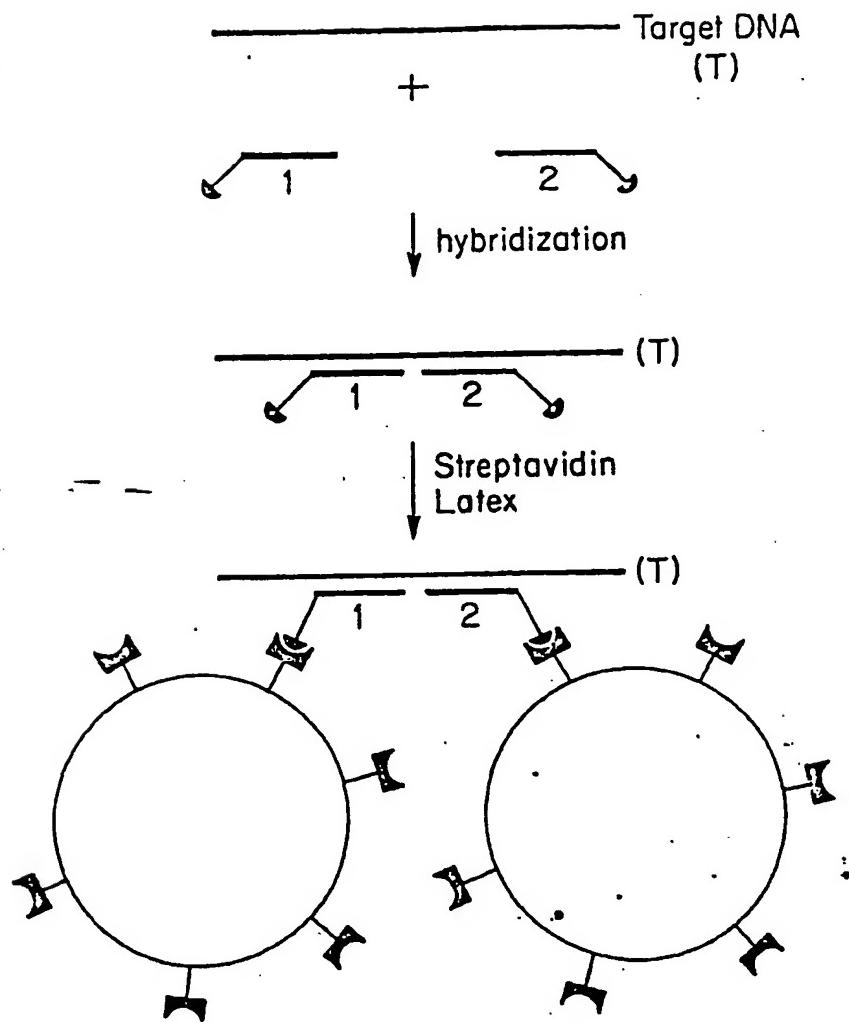
 Biotin Streptavidin

Fig. 1

2/3

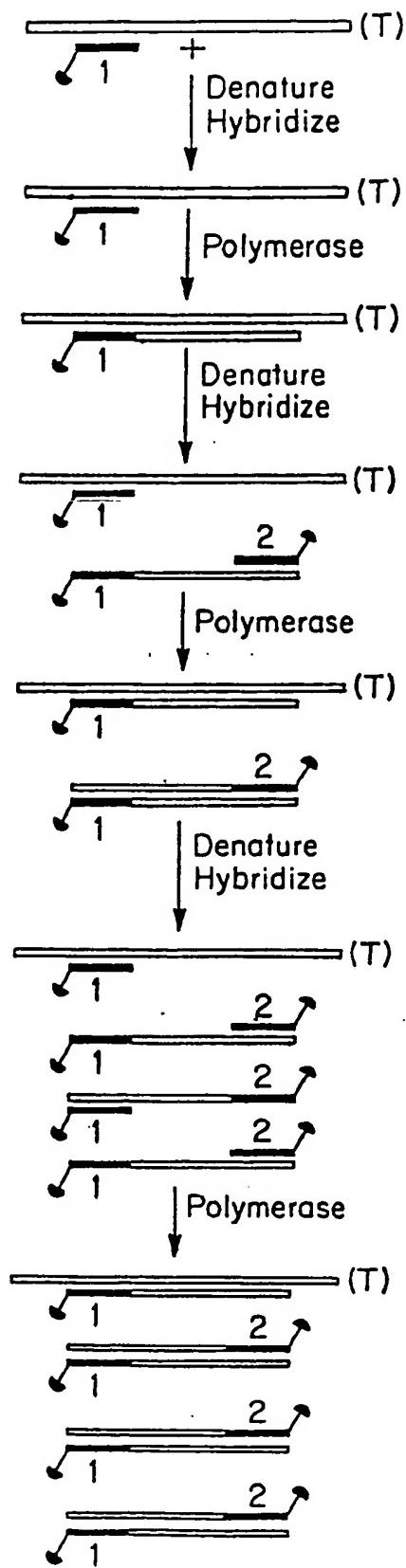


Fig. 2A

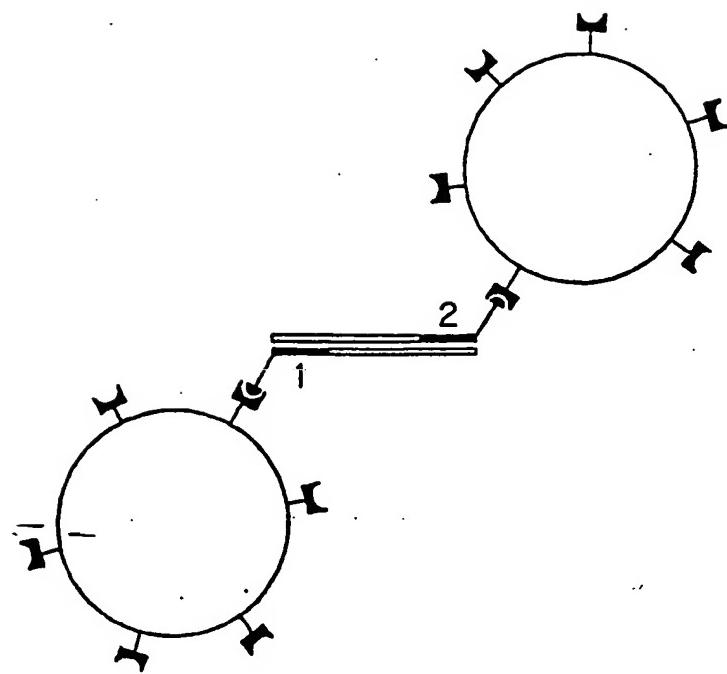


Fig. 2B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/03624

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁵: C 12 Q 1/68, G 01 N 33/543, G 01 N 33/553, G 01 N 33/546

II. FIELDS SEARCHED

Minimum Documentation Searched †

Classification System	Classification Symbols
IPC ⁵	C 12 Q, G 01 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ‡

III. DOCUMENTS CONSIDERED TO BE RELEVANT §

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ††	Relevant to Claim No. †‡
X	EP, A, 0139489 (ORTHO DIAGNOSTIC SYSTEMS) 2 May 1985 see the whole document	1-3, 6, 7, 12-14, 16, 17, 21, 22, 25, 26, 28, 31
X	EP, A, 0192168 (MOLECULAR DIAGNOSTICS INC.) 27 August 1986 see page 5, lines 2-13; page 8, lines 9-37; figure	1-7, 12-17, 21-26, 28-31
Y	--	1-10, 12-26, 28-31
Y	WO, A, 86/05815 (GENETICS INTERNATIONAL INC.) 9 October 1986 see page 6, line 8 - page 7, line 1; figure 1; page 14, line 20 - page 17, line 21; page 18, example 1	1-10, 12-26, 28-31
X	--	8, 18 ./.

* Special categories of cited documents: ††

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search
18th December 1989

Date of Mailing of this International Search Report
25.01.90

International Searching Authority

EUROPEAN-PATENT OFFICE

Signature of Authorized Officer

T.K. WILLIS

III DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	WO, A, 88/01374 (ANGENICS INC.) 25 February 1988 see the whole document -----	9,10

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US 8903624
SA 30876

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 16/01/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0139489	02-05-85	AU-A-	3355484	11-09-86
		CA-A-	1256005	20-06-89
		JP-A-	60093355	25-05-85
EP-A- 0192168	27-08-86	AU-A-	5329486	28-08-86
		JP-A-	61195699	29-08-86
WO-A- 8605815	09-10-86	AU-A-	5667186	23-10-86
		EP-A-	0216844	08-04-87
WO-A- 8801374	25-02-88	None		

DAVID F. ZINGER
CRAIG C. GROSETH
MICHAEL L. TOMPKINS
TODD P. BLAKELY
GARY J. CONNELL
SABRINA C. STAVISH
JOSEPH E. KOVARIK
SUSAN PRYOR WILLSON
LEWIS D. HANSEN
ROBERT R. BRUNELLI
DOUGLAS W. SWARTZ
RICHARD L. HUGHES
BRUCE A. KUGLER
DANA HARTIE CARDWELL
PAUL S. HANSRA
BENJAMIN B. LIEB
BRADLEY M. KNEPPER
MIRIAM DRICKMAN TRUDELL

PATENTS
TRADEMARKS
COPYRIGHTS

KERMITH F. ROSS
1910-1986

OF COUNSEL
PHILIP H. SHERIDAN
WANNELL M. CROOK
GILBERT E. ALBERDING*
THERESA A. BROWN

*MI, AL, IN Bar Only

TECHNICAL SPECIALISTS
DENNIS J. DUPRAY, Ph.D.
ANGELA DALLAS-PEDRETTI, Ph.D.

SHERIDAN ROSS
A Professional Corporation
ATTORNEYS AND COUNSELORS AT LAW
1560 BROADWAY
SUITE 1200
DENVER, COLORADO 80202-5141
TELEPHONE (303) 863-9700
FACSIMILE (303) 863-0223
E-MAIL srlaw@sheridanross.com

August 10, 2000

Dr. Chad A. Mirkin
Associate Professor - Department of Chemistry
Northwestern University
2145 Sheridan Road
Evanston, IL 60208-3113

Re: New PCT Application Corresponding to U.S. Patent Application No. 09/344,667 Entitled "NANOPARTICLES HAVING OLIGONUCLEOTIDES ATTACHED THERETO AND USES THEREFOR" in the Name of MIRKIN, Chad A. et al.; Our File No. 4149-1-1-1-PCT

Dear Dr. Mirkin:

As instructed, we have initiated a PCT application for the above-identified invention. We are now required to submit the enclosed Powers of Attorney. Please have these documents signed and dated on the lines indicated and return them to us as soon as possible in the enclosed, self-addressed stamped envelope.

Should you have any questions, please let us know. Thank you.

Best regards,

Sincerely,

SHERIDAN ROSS P.C.



Susan P. Willson

SPW/jmm
Enclosures

POWER OF ATTORNEY CONCERNING A GIVEN INTERNATIONAL APPLICATION

Patent Cooperation Treaty
Appointment of Agent or Common Representative

The undersigned applicant hereby appoints

Wannell M. Crook
Registration No. 31,071
and

David F. Zinger	Reg. No. 29,127
Craig C. Groseth	Reg. No. 31,713
Michael L. Tompkins	Reg. No. 30,980
Todd P. Blakely	Reg. No. 31,328
Gary J. Connell	Reg. No. 32,020
Sabrina Crowley Stavish	Reg. No. 33,374
Lewis D. Hansen	Reg. No. 35,536
Joseph E. Kovarik	Reg. No. 33,005
Douglas W. Swartz	Reg. No. 37,739
Bruce A. Kugler	Reg. No. 38,942
Robert R. Brunelli	Reg. No. 39,617
Riehard L. Hughes	Reg. No. 31,264
Tejpal S. Hansra	Reg. No. 38,172
Dana L. Hartje	Reg. No. 40,638
Angela Dallas-Pedretti	Reg. No. 42,460
Benjamin B. Lieb	Reg. No. 42,801
Bradley M. Knepper	Reg. No. 44,189
Theresa A. Brown	Reg. No. 32,547
Miriam R. Drickman	Reg. No. 42,499
Dennis J. DuPray	Reg. No. 46299

of Sheridan Ross P.C., 1560 Broadway, Suite 1200, Denver, Colorado 80202-5141 as agents to represent the undersigned before all the competent International Authorities in connection with the international application entitled "NANOPARTICLES HAVING OLIGONUCLEOTIDES ATTACHED THERETO AND USES THEREFOR", Attorney Docket No. 4149-1-1-1-PCT, filed with U.S. Receiving Office and to make or receive payments on their behalf.

APPLICANT: MIRKIN, Chad A.
111 16th St.
Wilmette, Illinois 60091
United States of America

Date: _____ By: _____
Chad A. Mirkin

**POWER OF ATTORNEY CONCERNING A GIVEN
INTERNATIONAL APPLICATION**

Patent Cooperation Treaty
Appointment of Agent or Common Representative

The undersigned applicant hereby appoints

Wannell M. Crook
Registration No. 31,071
and

David F. Zinger	Reg. No. 29,127
Craig C. Groseth	Reg. No. 31,713
Michael L. Tompkins	Reg. No. 30,980
Todd P. Blakely	Reg. No. 31,328
Gary J. Connell	Reg. No. 32,020
Sabrina Crowley Stavish	Reg. No. 33,374
Lewis D. Hansen	Reg. No. 35,536
Joseph E. Kovarik	Reg. No. 33,005
Douglas W. Swartz	Reg. No. 37,739
Bruce A. Kugler	Reg. No. 38,942
Robert R. Brunelli	Reg. No. 39,617
Richard L. Hughes	Reg. No. 31,264
Tejpal S. Hansra	Reg. No. 38,172
Dana L. Hartje	Reg. No. 40,638
Angela Dallas-Pedretti	Reg. No. 42,460
Benjamin B. Lieb	Reg. No. 42,801
Bradley M. Knepper	Reg. No. 44,189
Theresa A. Brown	Reg. No. 32,547
Miriam R. Drickman	Reg. No. 42,499
Dennis J. DuPray	Reg. No. 46,299

of Sheridan Ross P.C., 1560 Broadway, Suite 1200, Denver, Colorado 80202-5141 as agents to represent the undersigned before all the competent International Authorities in connection with the international application entitled "NANOPARTICLES HAVING OLIGONUCLEOTIDES ATTACHED THERETO AND USES THEREFOR", Attorney Docket No. 4149-1-1-1-PCT, filed with U.S. Receiving Office and to make or receive payments on their behalf.

APPLICANT: LETSINGER, Robert L.
 316 Third Street
 Wilmette, Illinois 60091
 United States of America

Date: _____

By: _____
Robert L. Letsinger,

**POWER OF ATTORNEY CONCERNING A GIVEN
INTERNATIONAL APPLICATION**

**Patent Cooperation Treaty
Appointment of Agent or Common Representative**

The undersigned applicant hereby appoints

**Wannell M. Crook
Registration No. 31,071
and**

David F. Zinger	Reg. No. 29,127
Craig C. Groseth	Reg. No. 31,713
Michael L. Tompkins	Reg. No. 30,980
Todd P. Blakely	Reg. No. 31,328
Gary J. Connell	Reg. No. 32,020
Sabrina Crowley Stavish	Reg. No. 33,374
Lewis D. Hansen	Reg. No. 35,536
Joseph E. Kovarik	Reg. No. 33,005
Douglas W. Swartz	Reg. No. 37,739
Bruce A. Kugler	Reg. No. 38,942
Robert R. Brunelli	Reg. No. 39,617
Richard L. Hughes	Reg. No. 31,264
Tejpal S. Hansra	Reg. No. 38,172
Dana L. Hartje	Reg. No. 40,638
Angela Dallas-Pedretti	Reg. No. 42,460
Benjamin B. Lieb	Reg. No. 42,801
Bradley M. Knepper	Reg. No. 44,189
Theresa A. Brown	Reg. No. 32,547
Miriam R. Drickman	Reg. No. 42,499
Dennis J. DuPray	Reg. No. 46,299

of Sheridan Ross P.C., 1560 Broadway, Suite 1200, Denver, Colorado 80202-5141 as agents to represent the undersigned before all the competent International Authorities in connection with the international application entitled "NANOPARTICLES HAVING OLIGONUCLEOTIDES ATTACHED THERETO AND USES THEREFOR", Attorney Docket No. 4149-1-1-1-1-PCT, filed with U.S. Receiving Office and to make or receive payments on their behalf.

APPLICANT: MUCIC, Robert C.
 1600 Bel Aire Dr.
 Glendale, California 91201
 United States of America

Date: _____

By: _____

Robert C. Mucic

**POWER OF ATTORNEY CONCERNING A GIVEN
INTERNATIONAL APPLICATION**

Patent Cooperation Treaty
Appointment of Agent or Common Representative

The undersigned applicant hereby appoints

Wannell M. Crook
Registration No. 31,071
and

David F. Zinger	Reg. No. 29,127
Craig C. Groseth	Reg. No. 31,713
Michael L. Tompkins	Reg. No. 30,980
Todd P. Blakely	Reg. No. 31,328
Gary J. Connell	Reg. No. 32,020
Sabrina Crowley Stavish	Reg. No. 33,374
Lewis D. Hansen	Reg. No. 35,536
Joseph E. Kovarik	Reg. No. 33,005
Douglas W. Swartz	Reg. No. 37,739
Bruce A. Kugler	Reg. No. 38,942
Robert R. Brunelli	Reg. No. 39,617
Richard L. Hughes	Reg. No. 31,264
Tejpal S. Hansra	Reg. No. 38,172
Dana L. Hartje	Reg. No. 40,638
Angela Dallas-Pedretti	Reg. No. 42,460
Benjamin B. Lieb	Reg. No. 42,801
Bradley M. Knepper	Reg. No. 44,189
Theresa A. Brown	Reg. No. 32,547
Miriam R. Drickman	Reg. No. 42,499
Dennis J. DuPray	Reg. No. 46,299

of Sheridan Ross P.C., 1560 Broadway, Suite 1200, Denver, Colorado 80202-5141 as agents to represent the undersigned before all the competent International Authorities in connection with the international application entitled "NANOPARTICLES HAVING OLIGONUCLEOTIDES ATTACHED THERETO AND USES THEREFOR", Attorney Docket No. 4149-1-1-1-1-PCT, filed with U.S. Receiving Office and to make or receive payments on their behalf.

APPLICANT: STORHOFF, James J.
 2121 Ridge Ave., Apt. 2J
 Evanston, Illinois 60201
 United States of America

Date: _____

By: _____

James J. Storhoff

**POWER OF ATTORNEY CONCERNING A GIVEN
INTERNATIONAL APPLICATION**

**Patent Cooperation Treaty
Appointment of Agent or Common Representative**

The undersigned applicant hereby appoints

Wannell M. Crook
Registration No. 31,071
and

David F. Zinger	Reg. No. 29,127
Craig C. Groseth	Reg. No. 31,713
Michael L. Tompkins	Reg. No. 30,980
Todd P. Blakely	Reg. No. 31,328
Gary J. Connell	Reg. No. 32,020
Sabrina Crowley Stavish	Reg. No. 33,374
Lewis D. Hansen	Reg. No. 35,536
Joseph E. Kovarik	Reg. No. 33,005
Douglas W. Swartz	Reg. No. 37,739
Bruce A. Kugler	Reg. No. 38,942
Robert R. Brunelli	Reg. No. 39,617
Richard L. Hughes	Reg. No. 31,264
Tejpal S. Hansra	Reg. No. 38,172
Dana L. Hartje	Reg. No. 40,638
Angela Dallas-Pedretti	Reg. No. 42,460
Benjamin B. Lieb	Reg. No. 42,801
Bradley M. Knepper	Reg. No. 44,189
Theresa A. Brown	Reg. No. 32,547
Miriam R. Drickman	Reg. No. 42,499
Dennis J. DuPray	Reg. No. 46,299

of Sheridan Ross P.C., 1560 Broadway, Suite 1200, Denver, Colorado 80202-5141 as agents to represent the undersigned before all the competent International Authorities in connection with the international application entitled "NANOPARTICLES HAVING OLIGONUCLEOTIDES ATTACHED THERETO AND USES THEREFOR", Attorney Docket No. 4149-1-1-1-1-PCT, filed with U.S. Receiving Office and to make or receive payments on their behalf.

APPLICANT: ELGHANIAN, Robert
8503 W. Catherine Avenue, Apt. 602
Chicago, Illinois 60656
United States of America

Date: _____ By: _____

Robert Elghanian

**ATTORNEY CONCERNING A GIVEN
INTERNATIONAL APPLICATION**

**Patent Cooperation Treaty
Appointment of Agent or Common Representative**

The undersigned applicant hereby appoints

Wannell M. Crook
Registration No. 31,071
and

David F. Zinger	Reg. No. 29,127
Craig C. Groseth	Reg. No. 31,713
Michael L. Tompkins	Reg. No. 30,980
Todd P. Blakely	Reg. No. 31,328
Gary J. Connell	Reg. No. 32,020
Sabrina Crowley Stavish	Reg. No. 33,374
Lewis D. Hansen	Reg. No. 35,536
Joseph E. Kovarik	Reg. No. 33,005
Douglas W. Swartz	Reg. No. 37,739
Bruce A. Kugler	Reg. No. 38,942
Robert R. Brunelli	Reg. No. 39,617
Richard L. Hughes	Reg. No. 31,264
Tejpal S. Hansra	Reg. No. 38,172
Dana L. Hartje	Reg. No. 40,638
Angela Dallas-Pedretti	Reg. No. 42,460
Benjamin B. Lieb	Reg. No. 42,801
Bradley M. Knepper	Reg. No. 44,189
Theresa A. Brown	Reg. No. 32,547
Miriam R. Drickman	Reg. No. 42,499
Dennis J. DuPray	Reg. No. 46,299

of Sheridan Ross P.C., 1560 Broadway, Suite 1200, Denver, Colorado 80202-5141 as agents to represent the undersigned before all the competent International Authorities in connection with the international application entitled "NANOPARTICLES HAVING OLIGONUCLEOTIDES ATTACHED THERETO AND USES THEREFOR", Attorney Docket No. 4149-1-1-1-PCT, filed with U.S. Receiving Office and to make or receive payments on their behalf.

APPLICANT: TATON, Thomas Andrew
 1368 West Greenleaf, Apt. 1S
 Chicago, IL 60626
 United States of America

Date: _____

By: _____

Thomas Andrew Taton